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Core binding factor β—smooth muscle myosin heavy chain chimeric protein involved in acute myeloid leukemia forms unusual nuclear rod-like structures in transformed NIH 3T3 cells

CISCA WIJMENGA*, PAULA E. GREGORY*, AMITAV HAJRA*, EVELIN SCHRÖCK†, THOMAS RIED†, ROLAND EILS‡, P. PAUL LIU*, AND FRANCIS S. COLLINS*§

*Laboratory of Gene Transfer and †Diagnostic Development Branch, National Center for Human Genome Research, 49 Convent Drive, Bethesda, MD 20892-4470; and ‡Interdisciplinary Center for Scientific Computing, University of Heidelberg, Heidelberg, Germany

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ABSTRACT **Patients with the M4Eo subtype of acute myeloid leukemia almost invariably are found to have an inversion of chromosome 16 in their leukemic cells, which results in a gene fusion between the transcription factor called core binding factor β (CBFβ) on 16q and a smooth muscle myosin heavy chain (SMMHC) gene on 16p. Subcellular localizations of the wild-type CBFβ and the CBFβ-SMMHC fusion protein were determined by immunofluorescence of NIH 3T3 cells that overexpress wild-type or fusion protein. Normal CBFβ showed an unexpected perinuclear pattern consistent with primary localization in the Golgi complex. The CBFβ-SMMHC fusion protein had a very different pattern. Nuclear staining included rod-like crystalline structures as long as 11 μm. The heterodimeric partner of CBFβ, CBFα, formed part of this complex. Cytoplasmic staining included stress fibers that colocalized with actin, probably as a consequence of the myosin heavy chain component of the fusion protein. Deletion of different regions of the CBFβ portion of the fusion protein showed that binding to CBFα was not required for nuclear translocation. However, deletion of parts of the SMMHC domain of the fusion protein involved in myosin-mediated filament formation resulted in proteins that did not form rod-like structures. These observations confirm previous indirect evidence that the CBFβ-SMMHC fusion protein is capable of forming macromolecular nuclear aggregates and suggests possible models for the mechanism of leukemic transformation.**

The core binding factor β–smooth muscle myosin heavy chain fusion protein (CBFβ-SMMHC) involved in acute myeloid leukemia subtype M4Eo arises from a pericentric inversion of chromosome 16 (1). The 165 N-terminal amino acids are encoded by the *CBFB* gene on 16q22, which normally forms one of the two components of the transcription factor core binding factor (CBF), also known as PEBP2. The C-terminal part of the fusion protein is a portion of the tail region of the SMMHC, encoded by the *MYH11* gene on 16p13. The CBFβ-SMMHC fusion protein (inv16 protein) has a molecular mass (relative mobility) of 67 kDa to 107 kDa, dependent upon the position of the breakpoint in the *MYH11* gene (for review, see ref. 2).[1]

The CBF/PEBP2 heterodimeric protein was originally identified by virtue of its binding to a conserved sequence found in the Moloney murine leukemia virus (Mo-MLV) and polyomavirus enhancers (3, 4). To date, CBF binding sites have been discovered in enhancers/promoters of various hematopoietic genes (5–7). CBF is a heterodimer consisting of a DNA-binding α subunit and a β subunit that increases the affinity of the α subunit for DNA. To date, three different α-subunit genes have been identified (8–12); in contrast, only a single β-subunit gene has been identified (1, 13, 14).

The mechanism of oncogenic transformation of hematopoietic cells by CBFβ-SMMHC is unknown. In addition to the requirement to bind CBFα, the presence of the myosin tail appears to be crucial for the ability to transform NIH 3T3 cells (15). As shown by Hajra *et al.* (15), the myosin tail region of CBFβ-SMMHC can assemble into multimers, presumably in the same way normal smooth muscle myosin molecules do. However, the presence of large complexes that are unable to migrate into a nondenaturing polyacrylamide gel suggests that the filaments can aggregate into much larger arrays.

To better understand the role of CBFβ-SMMHC in oncogenic transformation of NIH 3T3 cells, immunohistochemical studies were performed to study the cellular distribution of CBFβ-SMMHC in comparison to wild-type CBFβ. The effect of different deletion mutants derived from CBFβ-SMMHC was also studied. CBFβ was localized to the Golgi and nucleus of NIH 3T3 cells. In contrast, a large fraction of CBFβ-SMMHC forms novel rod-like structures in the nucleus; furthermore, the protein is also incorporated into nonmuscle myosin filaments that interact with actin to form filamentous structures in the cytoplasm.

MATERIALS AND METHODS

Cells and Cell Culture. Cell lines C182 and C/M are derivatives of NIH 3T3 cells stably transfected with expression constructs containing the full-length CBFB and CBFB-MYH11 cDNA, respectively (15). CBFB-MYH11 cDNA encodes the most common fusion protein (type A) observed in patients with inv(16) (for review, see ref. 2); the SMMHC isoform present in this construct is the 200-kDa isoform. Cell lines expressing mutant CBFβ-SMMHC constructs (C165, CΔex3/M, CΔC78/M, and C/MΔC68) have been described (15). Mutant constructs with deletions in the SMMHC portion of the fusion protein (C/MΔN202, C/MΔN253, and C/MΔN306) will be described elsewhere (A.H., unpublished data).

Antibodies. CBFβ polyclonal antiserum was generated against a CBFβ-GST-22.0 fusion protein, where GST is glutathione *S*-transferase (16). The CBFβ monoclonal antibody (mAb) anti-β14.1 has been described (15). Anti-CBFα is raised against the runt domain of CBFα; this polyclonal antibody is predicted to recognize all three CBFα proteins (16). Polyclonal antisera specific for the 200-kDa isoform (designated GPPP)

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Abbreviations: CBF, core binding factor; Mo-MLV, Moloney murine leukemia virus; SMMHC, smooth muscle myosin heavy chain; mAb, monoclonal antibody; 3-D, three dimensional; FITC, fluorescein isothiocyanate.

§To whom reprint requests should be addressed at: National Center for Human Genome Research, Building 31, Room 4B09, 31 Center Drive, Bethesda, MD 20892-2152.

of SMMHC was generated as described (15). Monoclonal antibody M3A5 (17) recognizes β-Cop, a peripheral 110-kDa Golgi membrane protein. Anti-actin mAb MAB1501 was obtained from Chemicon. An antibody against the inv(16) type A CBFβ-SMMHC chimeric junction (anti-Inv16A) was produced by immunizing rabbits with peptide J42723 (residues 157–174 of type A CBFβ-SMMHC (1) in single-letter amino acid code: DRSHREEMEVHELEKSKR; Lofstrand Laboratories, Gaithersburg, MD).

Immunoprecipitation and Immunoblot (Western) Analysis. Protein expression was determined by immunoprecipitation according to standard protocols (18). Nuclear and cytoplasmic protein fractions from the various transfected cell lines were immunoprecipitated by using protein A anti-CBFβ-conjugated beads. Immune complexes were separated by SDS/12% PAGE and transferred to nitrocellulose (Schleicher & Schuell) by standard procedures. Membranes were blocked for 1 h in 5% nonfat dry milk prior to 1 h of incubation with the CBFβ-specific mAb anti-β14 (1:10 dilution). The labeled bands were visualized by using the enhanced chemiluminescence (ECL; Amersham) protocol.

Immunofluorescence, Confocal Laser Scanning Microscopy and Three Dimensional (3-D) Reconstruction. Cells cultured on coverslips were rinsed with phosphate-buffered saline PBS and then fixed with absolute methanol at −20°C for 7 min. Immunofluorescence staining was as described (19). Bound antibody was visualized by using fluorescein isothiocyanate (FITC; Boehringer Mannheim) second antibodies. Where indicated, anti-CBFβ antiserum was blocked by preincubation with 10 μg of CBFβ-GST-22.0 protein. Double labeling experiments were identical to that described above except that both primary and secondary antibodies were coincubated. To detect mAbs Texas red-conjugated goat anti-mouse antibodies (Amersham) were used. Photographs were taken with a Zeiss microscope using Ektachrome 200 film.

Light optical sectioning of C/M nuclei stained with anti-Inv16A was performed with a confocal laser scanning fluorescence microscope (Leica TCS 4D, Heidelberg, Germany). The signals from CBFβ-SMMHC (FITC fluorescence; excitation wavelength, 488 nm), and the counterstaining of the cell (propidium iodide fluorescence; excitation wavelength, 568 nm) were captured simultaneously with a ×100/n.a. 1.4 objective. The distance in the *z* direction between the subsequent light optical sections was ≈300 nm, resulting in an image stack of 15–25 sections per nucleus. The size of the rod-like structures in *x*-, *y*- and *z* directions was measured by using the Leica TCS software. For 3-D reconstruction of individual light optical sections, the data volumes were transferred to a Silicon Graphics workstation (SGI IRIS INDIGO 2, Extreme Graphics). A new approach for simultaneous truly 3-D segmentation and determination of stereological parameters was applied (20). This procedure is based on the construction of Voronoi diagrams by dissection of the image into convex polyhedra ("Voronoi cells"). The Voronoi diagram of the stack of images in the FITC channel consisted of up to 27,000 Voronoi cells. After the tessellation step, each of the rod-like structures formed by the signals from CBFβ-SMMHC were segmented in the Voronoi diagram by using an interactively chosen threshold. Typically, each rod consisted of 5000–7000 Voronoi cells. The lower limit of the size (volume) of each Voronoi cell was chosen to be 1.5×10^{-20} m^3 which is below the smallest unit of resolution (2×10^{-20} m^3) achievable with a confocal laser scanning microscope. The segmented signals were visualized by using ray tracing (20).

RESULTS

Subcellular Localization of Wild-Type CBFβ and the inv16 Protein. Immunofluorescence staining of NIH 3T3 cells was performed with anti-CBFβ. A large fraction of CBFβ showed a perinuclear structure typical of the Golgi complex, and the rest was localized diffusely throughout the nucleus (Fig. 1*A*). Double-labeling experiments using an antibody (M3A5) specific to the β-COP protein—a peripheral Golgi membrane protein—supported the conclusion that wild-type CBFβ is indeed associated with the Golgi complex (Fig. 2 *A* and *D*). The specificity of the staining was demonstrated by loss of signal when the CBFβ polyclonal antibody was blocked by preincubation with the CBFβ-GST-22.0 protein (Fig. 1*B*). Compared with the endogenous level, CBFβ is about 5-fold overexpressed in cell line C182 (ref. 15, and Fig. 3). Staining the C182 cells with anti-CBFβ yielded results quantitatively comparable to NIH 3T3 cells, with diffuse nuclear staining and discrete Golgi staining (Fig. 1*C*). This subcellular localization was confirmed by Western blot analysis (Fig. 3, lanes 1–4).

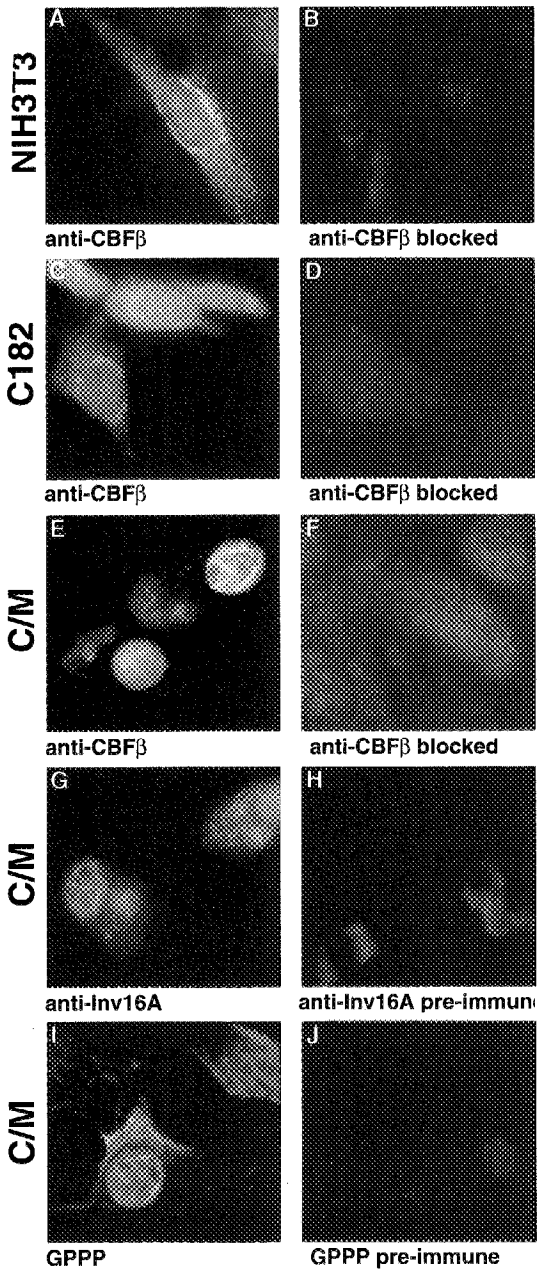

FIG. 1. Subcellular distribution of CBFβ and CBFβ-SMMHC proteins; these proteins were overexpressed in cell lines C182 and C/M, respectively. Cell lines and antibodies are as indicated. Specificity of the antibodies was determined by blocking the primary antibody with purified CBFβ protein (*B*, *D*, and *F*) or by using preimmune serum (*H* and *J*). C/M cells stained with anti-CBFβ were counterstained with propidium iodide.

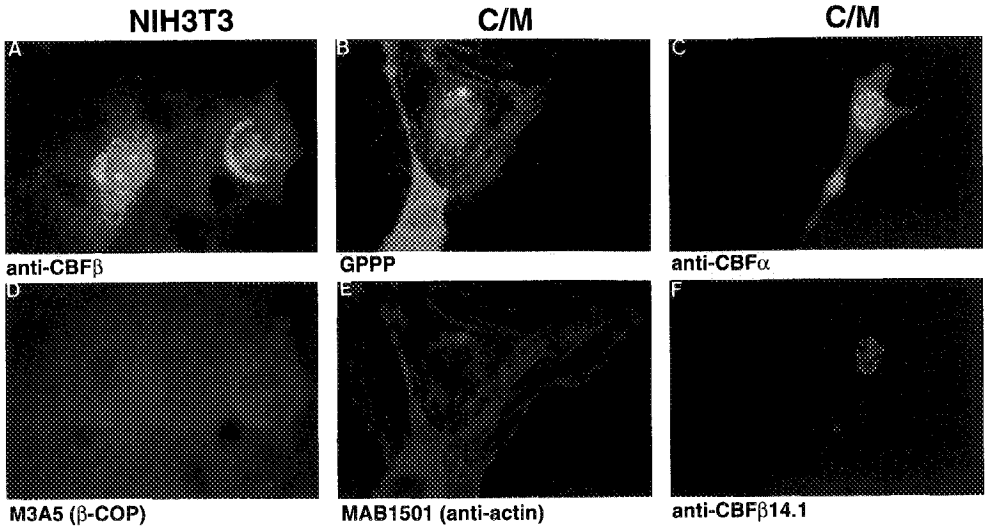

FIG. 2. (*A* and *D*) Colocalization of CBFβ with β-COP, a peripheral Golgi membrane protein. (*B* and *E*) Colocalization of CBFβ-SMMHC-positive stress fibers with actin. (*C* and *F*) CBFα colocalizes with the rod-shaped CBFβ-SMMHC structures.

C/M cells are NIH 3T3 cells that are stably transfected with *CBFB-MYH11* and express large amounts of CBFβ-SMMHC. One of the components of CBFβ-SMMHC, SMMHC, is normally not expressed in NIH 3T3 cells and, therefore, cannot be detected in untransfected cells by either immunofluorescence or Western blotting with antibody GPPP, which detects the C-terminal epitope of the 200-kDa isoform of SMMHC (data not shown). Immunofluorescence of C/M cells was performed with three different antibodies against the N-terminal (anti-CBFβ), the junction (anti-Inv16A), and the C-terminal (GPPP) portions of CBFβ-SMMHC. Approximately 90% of all cells show CBFβ-SMMHC to be present in the nucleus. The fusion protein assumes a variety of unusual nuclear structures, ranging from tiny speckles to large rod-shaped structures. The latter are found in about 40% of the cells, but the number of cells with rod-shaped structures increases with cell density. These structures are repeatedly seen with all three antibodies (Fig. 1 *E*, *G*, and *I*), demonstrating that they are specific for the CBFβ-SMMHC fusion protein. The specificity of the staining was confirmed by using preimmune serum (Fig. 1 *H* and *J*), or by preincubating with the purified CBFβ protein (Fig. 1*F*). Omission of the primary antibody resulted in no detectable staining. CBFβ-SMMHC is also present in the cytoplasm, in a pattern suggestive of

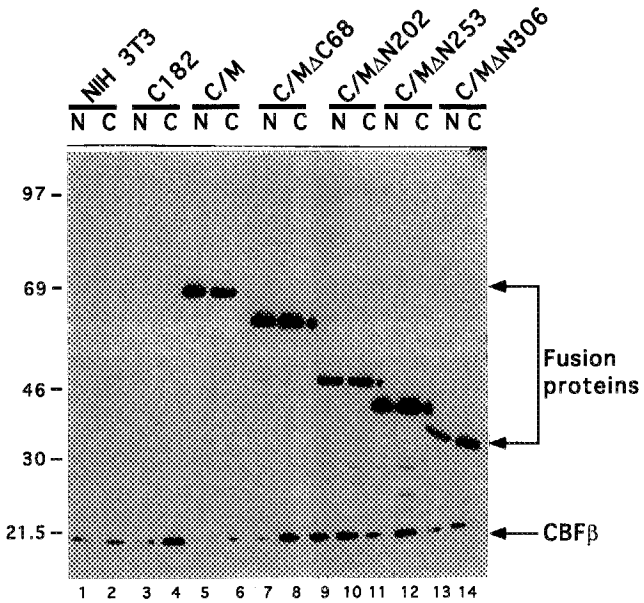

FIG. 3. Identification of CBFβ and CBFβ-SMMHC fusion proteins in nuclear (lanes N) and cytoplasmic (lanes C) fractions of NIH 3T3 cells and derivative cell lines. Cell lysates were immunoprecipitated with CBFβ antiserum; immune complexes were detected by using the monoclonal antibody anti-β14. Molecular weight standards (in kDa) are indicated. See Fig. 6 for a schematic representation of the different constructs.

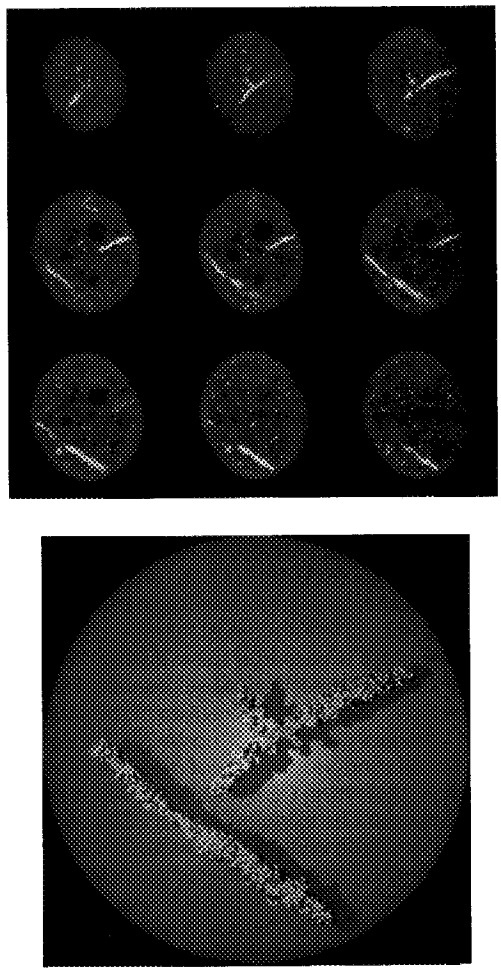

FIG. 4. (*Upper*) Light optical sections of a C/M cell obtained by confocal laser scanning microscopy demonstrate the presence of the CBFβ-SMMHC protein in contiguous sections which are 300 nm apart, implying a rod-like structure. (*Lower*) This was confirmed by 3-D reconstruction based on Voronoi diagrams. The CBFβ-SMMHC protein was detected with anti-Inv16A; the cells were counterstained with propidium iodide.

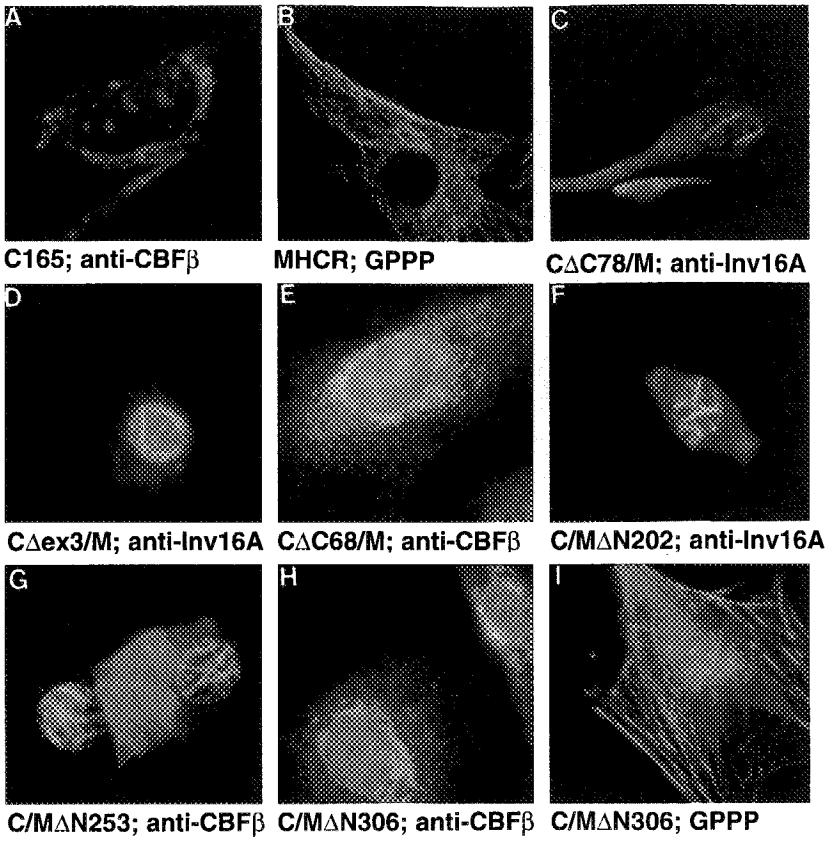

FIG. 5. Subcellular localization of mutant CBFβ-SMMHC proteins. Immunofluorescence labeling of CBFβ (with anti-CBFβ), or CBFβ-SMMHC (with anti-CBFβ, anti-Inv16A, or GPPP) proteins are as indicated. See Fig. 6 for a schematic representation of the different constructs.

cytoskeletal association. Double-labeling experiments with an anti-actin antibody show that CBFβ-SMMHC colocalizes with actin filaments (Fig. 2 *B* and *E*). The detection of stress-fibers seems to be antibody specific and can only be detected by using the GPPP antibody (compare Fig. 1 *E* and *G* to Fig. 1*I*), most likely determined by the accessibility of the epitope. The significance of this finding is unknown but presumably reflects the ability of CBFβ-SMMHC to interact with other nonmuscle myosins, as has been illustrated by using the yeast dihybrid system (C.W., unpublished data), and then in turn with actin. In agreement with the previous results, analysis of the nuclear and cytoplasmic protein fractions from cell line C/M by Western blot analysis shows a specific band with a molecular mass of 67 kDa in both fractions, which corresponds to the CBFβ-SMMHC chimeric protein (Fig. 3, lanes 5 and 6).

CBFβ can, in general, form a complex with CBFα. Although electrophoretic mobility shift assay (EMSA) experiments have previously demonstrated that the inv16 protein retains the ability to associate with CBFα (15), it was interesting to determine whether CBFα was associated with the rod-shaped nuclear structures formed by CBFβ-SMMHC. Double-labeling experiments using anti-CBFα and anti-β14.1 antibodies does in fact demonstrate that CBFα forms part of the rod-shaped structures observed in C/M cells (Fig. 2 *C* and *F*).

Confocal Laser Scanning Microscopy. Light optical sectioning with a confocal laser scanning microscope and 3-D reconstruction was applied to observe the structures of the CBFβ-SMMHC fusion protein in 12 C/M nuclei. About three to six rod-like shaped structures were found in about five consecutive sections in each nucleus. An example is shown in Fig. 4. Measurements of the length, width, and height of 12 different "rods" using the Leica TCS software revealed on average a length of 6.0 μm, a width of 0.45 μm, and a height of 2.5 μm. The presence of rod-shaped structures was confirmed by 3-D reconstruction based on the calculation of Voronoi diagrams. The results were visualized by ray tracing and exemplified in Fig. 4*B*.

Requirements for Nuclear Localization and Rod Formation. A series of CBFβ-SMMHC deletion mutants was also studied. Western blot analysis indicated variant proteins of the predicted size and expressed at similar levels as full-length CBFβ-SMMHC (Fig. 3, lanes 7–14; A.H., unpublished data). Representative immunofluorescence results are shown in Fig. 5, and a summary of the results from six different deletion constructs is shown in Fig. 6. Cell line C165—expressing only the CBFβ-portion of the CBFβ-SMMHC fusion protein—reveals the same staining pattern as wild-type CBFβ (Fig. 5*A*). Both CΔC78/M and CΔex3/M are truncated in the CBFβ portion of the CBFβ-SMMHC protein and have lost their ability to associate with CBFα (15). Nevertheless, these protein products were still able to enter the nucleus, where they exhibit the same rod-shaped structures as shown in C/M cells (Fig. 5 *C* and *D*). Cell lines expressing only the SMMHC part of the CBFβ-SMMHC protein (MHCR) or constructs truncated in the SMMHC part (C/MΔC68, C/MΔN202, C/MΔN253, and C/MΔN306) (Fig. 6), were analyzed. The protein expressed by cell line MHCR, which can only be detected by GPPP, was found exclusively in the cytoplasm (Fig. 5*B*) in a structure that colocalizes with actin (data not shown). C/MΔN202 and C/MΔN253 show a cytoplasmic and nuclear staining similar to the C/M cells (Fig. 5 *F* and *G*). However, no detectable staining was observed in cells overexpressing C/MΔC68 or C/MΔN306 when using anti-Inv16A (data not shown), presumably because of improper folding of these mutant proteins in such a way that the epitope is masked. Anti-CBFβ showed diffuse nuclear staining and a perinuclear localization comparable to CBFβ (Fig. 5 *E* and *H*). The GPPP antibody showed a diffuse nuclear staining and cytoplasmic stress fibers in C/MΔN306 cells (Fig. 5*I*). The protein expressed by C/MΔC68 cells lacks the epitope recognized by GPPP. Western blot analysis confirmed the presence of C/MΔN306 and C/MΔC68 proteins in both the nucleus and cytoplasm (Fig. 3, lanes 7, 8, 13, and 14). All staining data and previously determined information about association of deletion mutants with CBFα and their trans-

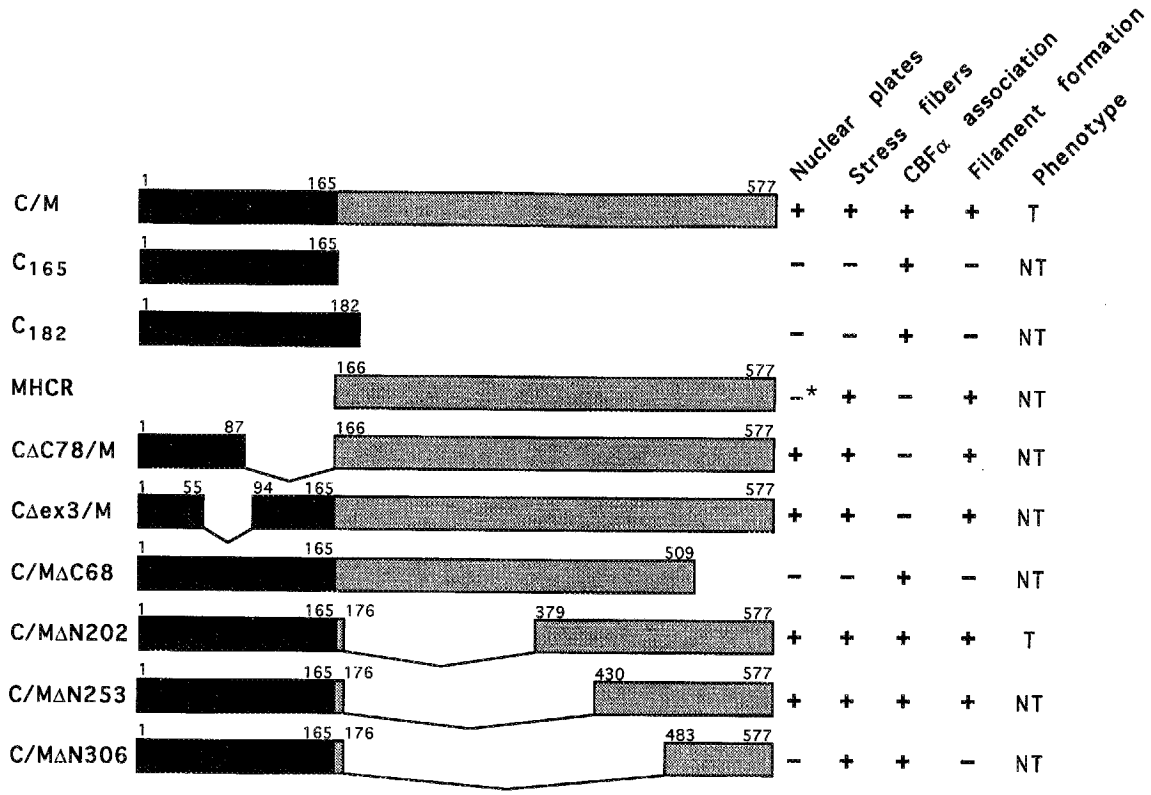

FIG. 6. Schematic representation of subcellular localization and functional characteristics of CBFβ and full-length or mutant CBFβ-SMMHC proteins. Data regarding the ability to associate with CBFα, to form multimers mediated by myosin, or to transform NIH 3T3 cells have been reported previously (ref. 15; also A.H., unpublished data). *, The protein expressed by the MHCR cell line is not found in the nucleus. T, transformed; NT, not transformed.

forming ability in NIH 3T3 cells (ref. 15; A.H., unpublished data) are summarized in Fig. 6.

DISCUSSION

This study shows that the β subunit of transcription factor CBF is not exclusively localized to the nucleus of NIH 3T3 cells. The nuclear CBFβ is expected to be accessible to bind to CBFα and participate in the formation of CBF–DNA complexes. In contrast, a large fraction of CBFβ is unexpectedly found to be associated with the Golgi complex; whether this extranuclear CBFβ represents a mechanism of sequestering CBFβ until activated by some unknown factor to translocate to the nucleus, as has been observed for the sterol regulatory element 1 binding protein (21) or the NF-κB/Rel family of transcription factors (22), needs further investigation.

Although the distribution of CBFβ-SMMHC between nucleus and cytoplasm is similar to CBFβ, the specifics of the compartmental localization are totally different. Approximately 40% of cells expressing CBFβ-SMMHC exhibit novel rod-shaped nuclear structures detectable by antisera against CBFβ, SMMHC, and the inv16 junction, clearly indicating that these structures contain the CBFβ-SMMHC fusion protein. Light optical sectioning of these cell nuclei using confocal laser scanning microscopy and 3-D reconstruction confirms a rod-like form of the CBFβ-SMMHC fusion. In addition, CBFα can be associated with these intranuclear structures as well. The number of nuclei exhibiting those rod-like structures increases to almost 70% when a higher density of cells is seeded onto a coverslip. Although the mechanism influencing this localization of inv16 protein is unclear, it could be influenced by cell–cell interactions.

Although it is known that the C terminus of myosin tails are capable of spontaneous assembly into bipolar thick filaments under polymerizing conditions, it was shown (23) that self-assembled filaments of smooth muscle myosin are side polar sheets; sheets may associate together to give a filament with a thicker cross section. It is likely that the CBFβ-SMMHC fusion protein behaves similarly and that the observed intranuclear structures are side polar sheets. The smaller "rods" and/or speckles that are also observed could reflect different assembly or disassembly structures involving the myosin portion of CBFβ-SMMHC. The conclusion that the formation of side polar sheets is mediated through the SMMHC portion of the fusion proteins is in concordance with the observations of cell lines C/MΔC68 and C/MΔN306. These cells do not show rod-shaped structures, and the fusion proteins expressed are also unable to form any type of multimers by electrophoretic mobility shift assay (ref. 15; also A.H., unpublished data).

The colocalization of CBFβ-SMMHC with actin filaments in the cytoplasm is probably the result of incorporation of CBFβ-SMMHC into nonmuscle myosin (NMM) filaments. The NMM–CBFβ-SMMHC complexes presumably do not interfere with normal NMM function. Although these complexes might be necessary for oncogenic transformation by some unknown mechanism, they are not sufficient alone, as shown by cell line C/MΔN306. This cell line also suggests that NMM–CBFβ-SMMHC complexes can form even when self-assembly of CBFβ-SMMHC into nuclear rods has been blocked by deletion of part of the myosin tail. The nuclear CBFβ-SMMHC structures are, presumably, the result of the assembly of native filaments, whereas in the cytoplasm the presence of other proteins like actin may influence the assembly process. Therefore, C/M cells might provide a mechanism to study myosin-mediated filament assembly *in vivo*.

The SMMHC-rod region of the fusion protein does not account for nuclear localization, since cell line MHCR reveals only cytoplasmic staining. Therefore, the CBFβ portion of the fusion protein has to be responsible for this process. Since wild-type CBFβ, as well as C165, CΔC78/M, and CΔex3/M, can

be translocated to the nucleus, a nuclear localization signal (NLS) has to be present within the first 55 amino acids of CBFβ. Wang *et al.* (13) reported the existence of a potential bipartite sequence between amino acids 19 and 35 that might serve as a NLS for CBFβ and, in the case of inv(16), also for CBFβ-SMMHC. However, Lu *et al.* (24) proposed recently that binding to CBFα is a requirement for nuclear localization of CBFβ. Constructs CΔC78/M and CΔex3/M lack the ability to associate with CBFα (15), but still form intranuclear structures. Hence, CBFα is not necessary for translocation of CBFβ-SMMHC to the nucleus. This observation seems to be in contrast to the results reported by Lu *et al.* (24), unless CBFβ and CBFβ-SMMHC use different mechanisms for nuclear localization.

The simplest mechanism responsible for CBFβ-SMMHC transformation is a dominant negative model, in which CBFβ-SMMHC interferes with normal CBF function. Although the rod-like structures sequester a substantial part of CBFα, thereby reducing the formation of normal CBF complexes, CBFα–CBFβ-SMMHC complexes most likely also interfere with CBF transactivation activities, as shown by the ability to repress transcription from the Mo-MLV enhancer and enhance transcription from the Rous sarcoma virus (RSV) long terminal repeat (LTR) (A.H., unpublished data). The formation of nuclear rod-like structures appears to be necessary for transformation but not sufficient (as exemplified by C/MΔN253). Since C/MΔN253 is also unable to repress the MoMLV enhancer or activate the RSV LTR (A.H., unpublished data), the CBFα–C/MΔN253 complex might not be large enough to interfere with transcription factor binding sites adjacent to the CBF binding site in either the Mo-MLV enhancer or the RSV LTR.

Thus, the formation of macromolecular aggregates of CBFβ-SMMHC, previously suspected by gel mobility-shift data, has been confirmed and visualized. It is perhaps surprising that such large nuclear structures are not damaging to cell viability. Though the NIH 3T3 cell has proved to be a useful model to study the transforming properties of the CBFβ-SMMHC fusion protein, it will also be helpful in the future to extend these studies to leukemic cells *in vivo*.

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